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РОССИЙСКОЕ АГЕНТСТВО ПО ПАТЕНТАМ И ТОВАРНЫМ ЗНАКАМ
(РОСПАТЕНТ)
ФЕДЕРАЛЬНЫЙ ИНСТИТУТ ПРОМЫШЛЕННОЙ СОБСТВЕННОСТИ

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Название изобретения METHOD FOR PRODUCING L-AMINO ACID USING BACTERIA BELONGING TO THE GENUS ESCHERICHIA

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METHOD FOR PRODUCING L-AMINO ACID USING BACTERIA BELONGING TO THE GENUS ESCHERICHIA

Technical field

The present invention relates to biotechnology, specifically to a method for producing L-amino acids by fermentation and more specifically to genes derived from bacteria *Escherichia coli*. The genes are useful for improvement of L-amino acid productivity, for example, L-threonine, L-valine, L-proline, L-leucine and L-methionine.

Background art

Conventionally the L-amino acids have been industrially produced by method of fermentation utilizing strains of microorganisms obtained from natural sources or mutant of the same especially modified to enhance L-amino acid productivity.

There have been disclosed many techniques to enhance L-amino acid productivity, for example, by transformation of microorganism by recombinant DNA (see, for example, US patent No. 4,278,765). These techniques based on the increasing of activities of the enzymes involved into amino acid biosynthesis and/or desensitizing the target enzymes to the feedback inhibition by produced L-amino acid (see, for example, Japanese Laid-open application No56-18596 (1981), WO 95/16042 or US patent Nos. 5,661,012 and 6,040,160).

On the other hand, increased L-amino acid excretion can enhance the productivity of strain producing L-amino acid. Strain of bacterium belonging to the genus *Corynebacterium* having increased expression of L-lysine excretion gene (*lysE* gene) is disclosed (WO 9723597A2). In addition, genes coding for the efflux proteins suitable for secretion of L-cysteine, L-cystine, N-acetylserine or thiazolidine derivatives are also disclosed (USA Patent No. 5,972,663).

At present several *Escherichia coli* genes coding for putative membrane proteins enhancing L-amino acid production are disclosed. Additional copy of *rhtB* gene makes a bacterium more resistant to L-homoserine and enhances production of L-homoserine, L-threonine, L-alanine, L-valine and L-isoleucine (European patent application EP994190A2). Additional copy of *rhtC* gene makes a bacterium more resistant to L-homoserine and L-threonine and enhances production of L-homoserine, L-threonine and L-leucine (European patent application EP1013765A1). Additional

copy of *yahN*, *yeaS*, *yfiK* and *yggA* genes enhance production of L-glutamic acid, L-lysine, L-threonine L-alanine, L-histidine, L-proline, L-arginine, L-valine and L-isoleucine (European patent application EP1016710A2). And though complete genome sequence of *Escherichia coli* strain K-12 is described (Blattner F.R., Plunkett G., Bloch C.A. et al., Science, 227, 1453-1474, 1997; ftp://ftp.genetics.wisc.edu/pub/sequence/ecolim52.seq.gz), there are many ORFs, the function of which still remain unknown.

Disclosure of the invention

An object of present invention is to enhance the productivity of L-amino acid producing strains and to provide a method for producing L-amino acid, for example, L-threonine, L-valine, L-proline, L-leucine or L-methionine, using the strains.

This aim was achieved by identifying genes coding for proteins, which are not involved into biosynthetic pathway of target L-amino acid but enhance its production. An example of such protein could be a membrane protein having L-amino acid excretion activity. Based on the analysis of complete genome sequence of *Escherichia coli,* proteins with 4 or more putative transmembrane segments (TMS) were selected. As a result of diligent screening, the present inventors have identified two genes among them, that is b2682 and b2683, and thoroughly studied it. The genes b2682 and b2683 have been known as putative CDS which may encode functionally unknown proteins (nucleotide numbers 92 to 829 and 819 to 1154 in the sequence of GenBank accession AE000353 U00096, respectively). The gene b2683 is also known as *ygaH*. Also the present inventors have found that by enhancing the activities of the proteins encoded by b2682 and b2683 genes the productivity of L-amino acid producing strain is enhanced. Thus the present invention has been completed.

The present inventions are as follows:

1). An L-amino acid producing bacterium belonging to the genus *Escherichia,* wherein L-amino acid production by the bacterium is enhanced by enhancing an activities of proteins as defined in the following (A) or (B), and (C) or (D) in a cell of the bacterium:

(A) a protein which comprises the amino acid sequence shown in SEQ ID NO:3 in Sequence listing;

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the

amino acid sequence shown in SEQ ID NO:3 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to the L-amino acids and/or its analogs;

(C) a protein which comprises the amino acid sequence shown in SEQ ID NO:5 in Sequence listing;

(D) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 5 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to the L-amino acids and/or its analogs;.

(hereinafter, the proteins as defined in the above (A) or (B) and (C) or (D) are referred to as "proteins of the present invention")

2). The bacterium according to the above bacterium, wherein the activities of the proteins as defined in (A) or (B) and (C) or (D) are enhanced by transformation of the bacterium with a DNA coding for the proteins as defined in (A) or (B) and (C) or (D), or by alteration of expression regulation sequence of said DNA on the chromosome of the bacterium.

3). The bacterium according to the above bacterium, wherein the transformation is performed with a multicopy vector.

4). A method for producing L-amino acid, which comprises cultivating the bacterium according to the above bacterium in a culture medium and collecting from the culture medium L-amino acid to be produced and accumulated.

5) The method according to the above method, wherein L-amino acid is L-threonine.

6). The method according to the above method, wherein the bacterium has enhanced expression of threonine operon.

7). The method according to the above method, wherein L-amino acid is L-valine.

8). The method according to the above method, wherein the bacterium has enhanced expression of *ilv* operon.

9). The method according to the above method, wherein L-amino acid is L-proline.

10). The method according to the above method, wherein the bacterium has enhanced expression of genes for proline biosynthesis.

11). The method according to the above method, wherein L-amino acid is L-leucine.

12). The method according to the above method, wherein the bacterium has enhanced expression of *leu* operon.

13). The method according to the above method, wherein L-amino acid is L-methionine.

14). The method according to the above method, wherein the bacterium has enhanced expression of *met* regulon.

The method for producing L-amino acid includes production of L-threonine using L-threonine producing bacterium wherein activities of the proteins of the present invention such as that comprising amino acid sequence shown in SEQ ID NO:3 and SEQ ID NO:5 are enhanced. Also a method for producing L-amino acid includes production of L-valine using L-valine producing bacterium wherein activities of the proteins of the present invention such as that comprising amino acid sequence shown in SEQ ID NO:3 and SEQ ID NO:5 are enhanced. Besides, method for producing L-amino acid includes production of L-proline using L-proline producing bacterium wherein activities of the proteins of the present invention such as that comprising amino acid sequence shown in SEQ ID NO:3 and SEQ ID NO:5 are enhanced. Moreover, method for producing L-amino acid includes production of L-leucine using L-leucine producing bacterium wherein activities of the proteins of the present invention such as that comprising amino acid sequence shown in SEQ ID NO:3 and SEQ ID NO:5 are enhanced. And, method for producing L-amino acid includes production of L-methionine using L-methionine producing bacterium wherein activities of the proteins of the present invention such as that comprising amino acid sequence shown in SEQ ID NO:3 and SEQ ID NO:5 are enhanced.

The present invention will be explained in detail below.

The bacterium of the present invention is an L-amino acid producing bacterium belonging to the genus *Escherichia,* wherein L-amino acid production by the bacterium is enhanced by enhancing an activities of the proteins of the present invention in a cell of the bacterium.

A bacterium of present invention is L-amino acid producing bacterium belonging to the genus *Escherichia* having enhanced activities of proteins, which enhance the productivity of the target L-amino acid. Concretely the bacterium of

present invention is L-amino acid producing bacterium belonging to the genus *Escherichia* which has enhanced activities of the proteins of the present invention. More concretely the bacterium of present invention harbors the DNA having overexpressed b2682 and b2683 genes on chromosomal DNA or plasmid in the bacterium and has enhanced ability to produce L-amino acid, for example L-threonine, L-valine, L-proline, L-leucine and L-methionine.

The proteins of the present invention include ones as defined in the following (A) or (B), and (C) or (D):

(A) a protein which comprises the amino acid sequence shown in SEQ ID NO:3 in Sequence listing;

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO:3 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to the L-amino acids and/or its analogs;

(C) a protein which comprises the amino acid sequence shown in SEQ ID NO5 in Sequence listing;

(D) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 5 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to the L-amino acids and/or its analogs.

The number of "several" amino acids differs depending on the position or the type of amino acid residues in the three-dimensional structure of the protein It may be 2 to 24, preferably 2 to 12, and more preferably 2 to 5 for the protein (A), and 2 to 11, preferably 2 to 7, and more preferably 2 to 5 for the protein (C), respectively.

Resistance to L-amino acids and/or its analogs means ability for bacterium to grow on a minimal medium containing L-amino acid or its analog in concentration under which the wild type or the parental strain of the bacterium cannot grow, or ability for bacterium to grow faster on a medium containing L-amino acid or its analog than the wild type or the parental strain of the bacterium. L-amino acid analogs are exemplified by 3,4-dihydroproline, DL-thiaisoleucine, DL-o-methylserine, 4-azaleucine, norleucine, L-o-fluorophenylalanine and DL-o-fluorophenylalanine. Above mentioned concentration of L-amino acid or its analog varies very significantly (from 0.5 μg/ml for DL-thiaisoleucine to 9600 μg/ml for DL-o-methylserine) depend on the structure of used compound. For example, such

concentration is generally 7 to 70 μg/ml, preferably 20 to 25 μg/ml in case of 3,4-dihydroproline; generally 0.5 to 5 μg/ml, preferably 0.9 to 1.1 in case of DL-thiaisoleucine; generally 1100 to 9600 μg/ml, preferably 3000 to 3500 in case of DL-o-methylserine; generally 15 to 150 μg/ml, preferably 40 to 50 μg/ml in case of 4-azaleucine; generally 150 to 1500 μg/ml, preferably 450 to 550 μg/ml in case of norleucine; generally 0.6 to 6 μg/ml, preferably 1.5 to 2 μg/ml in case of L-o-fluorophenylalanine; and generally 2 to 20 μg/ml, preferably 5 to 7 μg/ml in case of DL-o-fluorophenylalanine.

The bacterium of the present invention also includes one wherein the activities of the proteins of the present invention are enhanced by transformation of said bacterium with DNA coding for protein as defined in (A) or (B), and (C) or (D), or by alteration of expression regulation sequence of said DNA on the chromosome of the bacterium.

The DNA, which is used for modification of the bacterium of the present invention, codes for putative membrane protein. Concretely the DNA codes for protein having 4 or more transmembrane segments. Such DNA may code for proteins having L-amino acid excretion activity. More concretely, the DNA is represented by b2682 and b2683 genes. It is necessary to notice that coding region of b2682 gene at position 728-738 and coding region of b2683 gene at position 1-11 are overlapping. Both genes can be obtained by, for example, PCR using primers having nucleotide sequence shown in SEQ ID No: 1 and 2 as a single PCR product.

Analysis of complete genome sequence of *Escherichia coli* allowed to select the genes coding for proteins having 4 or more putative TMS. Proteins with known function and transporters described by Paulsen I.T., Sliwinski M.I., Saier M.H. (*J.Mol.Biol.*, 1998, 277, 573) and Linton K.J., Higgins C.F.(*Molecular Microbiology*, 1998, 28(1), 5) were excluded from the group to be screened. As a result of diligent screening among the rest of genes, several genes coding for putative membrane exporters were chosen. And it was found the overexpression of b2682 and b2683 genes enhances the L-amino acid production by L-amino acid producing strain.

The DNA of the present invention includes a DNA coding for the protein which include deletion, substitution, insertion or addition of one or several amino acids in one or more positions on the protein (A) or (C) as long as they do not lose the activity of the protein. Although the number of "several" amino acids differs

depending on the position or the type of amino acid residues in the three-dimensional structure of the protein, it may be 2 to 24, preferably 2 to 12, and more preferably 2 to 5 for the protein (A), and 2 to 11, preferably 2 to 7, and more preferably 2 to 5 for the protein (C), respectively. The DNA coding for substantially the same protein as the protein defined in (A) or (C) may be obtained by, for example, modification of nucleotide sequence coding for the protein defined in (A) or (C) using site-directed mutagenesis so that one or more amino acid residue will be deleted, substituted, inserted or added. Such modified DNA can be obtained by conventional methods using treatment with reagents and conditions generating mutations. Such treatment includes treatment the DNA coding for proteins of present invention with hydroxylamin or treatment the bacterium harboring the DNA with UV irradiation or reagent such as N-methyl-N'-nitro-N-nitrosoguanidine or nitrous acid.

The DNA of the present invention include variants which can be found in the different strains and variants of bacteria belonging to the genus *Escherichia* according to natural diversity. The DNA coding for such variants can be obtained by isolating the DNA, which hybridizes with b2862 or b2683 gene or part of the genes under the stringent conditions, and which codes the protein enhancing L-amino acid production. The term "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. For example, the stringent conditions includes a condition under which DNAs having high homology, for instance DNAs having homology no less than 70% to each other, are hybridized. Alternatively, the stringent conditions are exemplified by conditions which comprise ordinary condition of washing in Southern hybridization, e.g., 60°C, 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS. As a probe for the DNA which codes for variants and hybridizes with b2862 or b2683 gene, a partial sequence of the nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 5 respectively can also be used. Such a probe may be prepared by PCR using oligonucleotides produced based on the nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 5 as primers, and a DNA fragment containing the nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 5 as a template. When a DNA fragment in a length of about 300 bp is used as the probe, the conditions of washing for the hybridization consist of, for example, 50°C, 2 x SSC, and 0.1% SDS.

Transformation of bacterium with DNA coding for protein means introduction of the DNA into bacterium cell for example by conventional methods to increase

expression of the gene coding for the protein of present invention and to enhance the activity of the protein in the bacterial cell.

Techniques for enhancement of gene expression includes methods increasing the gene copy number. Introduction of a gene into a vector that is able to function in a bacterium belonging to the genus *Escherichia* increases copy number of the gene. For such purposes multi-copy vectors can be preferably used. The multi-copy vector is exemplified by pBR322, pMW119, pUC19, pET22b or the like.

Besides, enhancement of gene expression can be achieved by introduction of multiple copies of the gene into bacterial chromosome by, for example, method of homologous recombination or the like.

In case that expression of two or more genes is enhanced, the genes may be harbored together on the same plasmid or separately on different plasmids. It is also acceptable that one of the genes is harbored on a chromosome, and the other gene is harbored on a plasmid.

On the other hand, enhancement of gene expression can be achieved by locating the DNA of the present invention under control of a potent promoter. For example, *lac* promoter, *trp* promoter, *trc* promoter, P_L promoter of lambda phage are known as potent promoters. Using the potent promoter can be combined with multiplication of gene copies.

The bacterium of the present invention can be obtained by introduction of the aforementioned DNAs into bacterium inherently having ability to produce L-amino acid. Alternatively, the bacterium of present invention can be obtained by imparting ability to produce L-amino acid to the bacterium already harboring the DNAs. For the parent strain which is to be enhanced in activities of the proteins of the present invention, L-threonine producing bacteria belonging to the genus *Escherichia* such as strains VL2054 (VKPM B- 8067), VNIIGenetika 472T23 (US patent No.5,631,157), VKPM B-3996 (US patent Nos. 5,175,107 and 5,976,843), KCCM-10132 (WO009660A1), KCCM-10133 (WO009661A1) or the like can be employed. Also for the parent strain which is to be enhanced in activities of the proteins of the present invention, L-valine producing bacteria belonging to the genus *Escherichia* such as H-81 (VKPM B- 8066), NRRL B-12287 and NRRL B-12288 (US patent No. 4,391,907), VKPM B-4411 (US patent No. 5,658,766), VKPM B-7707 (European patent application EP1016710A2) or the like is employed. Besides, for the parent strain which is to be enhanced in activities of the proteins of the present invention, L-proline

producing bacteria belonging to the genus *Escherichia* such as NRRL B-12403 and NRRL B-12404 (GB2075056), VKPM B-8012 (Russian patent application 2000124295), plasmid mutants described in the patent DE3127361, plasmid mutants described by Bloom F.R. et al (The 15th Miami winter symposium, 1983, p.34) or the like are employed. Also, for the parent strain which is to be enhanced in activities of the proteins of the present invention, L-leucine producing bacteria belonging to the genus *Escherichia* such as H-9070 (FERM BP-4704) and H-9072 (FERM BP-4706) (US5744331), VKPM B-7386 and VKPM B-7388 (RU2140450), W1485atpA401/pMWdAR6, W1485lip2/pMWdAR6 and AJ12631/pMWdAR6 (EP0872547) or the like are employed. And, for the parent strain which is to be enhanced in activities of the proteins of the present invention, L-methionine producing bacteria belonging to the genus *Escherichia* such as AJ11539 (NRRL B-12399), AJ11540 (NRRL B-12400), AJ11541 (NRRL B-12401), AJ 11 542 (NRRL B-12402) (GB2075055) or the like are employed as well.

The bacterium of the present invention may be further enhanced expression of one or more genes which are involved in L-amino acid biosynthesis. Such genes are exemplified by threonine operon, which preferably comprises a gene encoding aspartate kinase – homoserine dehydrogenase of which feedback inhibition by L-threonine is desensitized (Japanse Patent Publicaion No. 1-29559), for L-threonine producing bacteria. Such genes are exemplified by *ilv* operon, i. e. *ilvGMEDA* operon, which does not preferably express threonine deaminase and of which attenuation is suppressed (Japanese Patent Laid-Open Publication No. 8-47397), for L-valine producing bacteria. Such genes are exemplified by genes for L-proline biosynthesis, which are preferably represented by gene *proB* encoding for glutamate kinase of which feedback inhibition by L-proline is desensitized (DE3127361), for L-proline producind bacteria. Also, such genes are exemplified by leucine operon, i. e. *leu* operon, which preferably comprises a gene coding for isopropylmalate synthase of which feedback inhibition by L-leucine is desensitized (Russian patent application 99114325), for L-leucine producing bacteria. Also, such genes are exemplified by methionine regulon, for L-methionine producing bacteria. The methionine regulon may have mutated genes coding for proteins lowered in activity in repressing the amino acid biosynthesis. Such gene is exemplified by variation type metJ gene coding for a L-methionine biosynthesis-relating repressor protein from *E. coli* of which activity in repressing methionine biosynthesis is lowered (JP2000157267A2).

The method of the present invention includes method for producing L-threonine, comprising steps of cultivating the bacterium of the present invention in a culture medium, to allow L-threonine to be produced and accumulated in the culture medium, and collecting L-threonine from the culture medium. Also the method of present invention includes method for producing L-valine, comprising steps of cultivating the bacterium of the present invention in a culture medium, to allow L-valine to be produced and accumulated in the culture medium, and collecting L-valine from the culture medium. Besides, the method of present invention includes method for producing L-proline, comprising steps of cultivating the bacterium of the present invention in a culture medium, to allow L-proline to be produced and accumulated in the culture medium, and collecting L-proline from the culture medium. Also, the method of present invention includes method for producing L-leucine, comprising steps of cultivating the bacterium of the present invention in a culture medium, to allow L-leucine to be produced and accumulated in the culture medium, and collecting L-leucine from the culture medium. And, the method of present invention includes method for producing L-methionine, comprising steps of cultivating the bacterium of the present invention in a culture medium, to allow L-methionine to be produced and accumulated in the culture medium, and collecting L-methionine from the culture medium.

In the present invention, the cultivation, the collection and purification of L-amino acid from the medium and the like may be performed in a manner similar to the conventional fermentation method wherein an amino acid is produced using a microorganism. A medium used for culture may be either a synthetic medium or a natural medium, so long as the medium includes a carbon source and a nitrogen source and minerals and, if necessary, appropriate amounts of nutrients which the microorganism requires for growth. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on the mode of assimilation of the used microorganism, alcohol including ethanol and glycerol may be used. As the nitrogen source, various ammonium salts such as ammonia and ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean-hydrolysate and digested fermentative microorganism are used. As minerals, potassium monophosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium chloride, and the like are used.

The cultivation is performed preferably under aerobic conditions such as a shaking culture, and stirring culture with aeration, at a temperature of 20 to 40 °C, preferably 30 to 38 °C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 5-day cultivation leads to the accumulation of the target L-amino acid in the liquid medium.

After cultivation, solids such as cells can be removed from the liquid medium by centrifugation or membrane filtration, and then the target L-amino acid can be collected and purified by ion-exchange, concentration and crystallization methods.

Brief description of the drawing

Figure 1 shows the construction of plasmid pΔlacZ.

Best Mode for Carrying out the Invention

The present invention will be more concretely explained below with reference to Examples. In the Examples an amino acid is of L-configuration unless otherwise noted.

Example 1: Cloning of the b2682 and b2683 genes on the plasmid pΔlacZ.

For cloning of the b2682 and b2683 genes vector pΔlacZ was used. Vector pΔlacZ is a derivative of the vector pET-22b(+) (Novagen, Madison, WI, USA). pET-22b(+) was treated by *Bgl*II and *Xba*I and ligated with polymerase chain reaction (PCR) fragment of plasmid pMB9-*lac* (Fuller F., *Gene*, 19, 43-54, 1982)treated with the same restrictases and carried P_{lac} UV5 promoter. For amplifying the P_{lac} UV5 promoter fragment by PCR primers having sequence depicted in SEQ ID Nos: 7 and 8 were used. The resulted plasmid was supplemented with structural part of *lacZ* gene (237 bp without promoter) by cloning *Sal*I-*Bam*HI fragment of the plasmid pJEL250 (Dymakova E. *et al.*, *Genetika* (rus), *35*, 2, 181-186, 1999). Scheme for obtaining vector pΔlacZ is shown in Figure 1.

The initial material for cloning of *E.coli* b2682 and b2683 putative reading frames (b2682 and b2683 genes) was the PCR fragment, which was obtained using DNA from *E. coli* strain TG1 as a template. For synthesis of this fragment two primers having sequence depicted in SEQ ID Nos: 1 and 2 were used. PCR was carried out on "Perkin Elmer GeneAmp PCR System 2400" under the following

conditions: 40 sec. at 95 °C, 40 sec. at 47 °C, 40 sec. at 72 °C, 30 cycles. Thus, the 1158 bp linear DNA fragment contained b2682 and b2683 genes was obtained. This PCR fragment was treated by *Xba*I and *Bam*HI restrictases and inserted into multicopy vector pΔlacZ previously treated by the same restrictases.

Resulted plasmid with the PCR fragment was named pYGAZH and carried both gene b2682 and b2683 under the control of the lactose promoter (P_{lac} UV5).

Example 2: The influence of the amplified b2682 and b2683 genes on resistance of *E. coli* strain TG1 to amino acids and its analogs.

E. coli strain TG1(pYGAZH) and TG1 strain having a vector without insertion (control strain) were grown overnight on LB medium supplemented with ampicilline (100 μg/ml). The night cultures of all strains were diluted at 25 times in fresh LB medium supplemented with ampicilline (100 μg/ml) and IPTG (0.5 mM) and were incubated 2 hours at 37 °C with aeration. The log phase cultures were diluted in 0,9% solution of NaCl and about 1000 cells were seeded on plates with solid Adams medium supplemented with ampicilline (100 μg/ml), IPTG (0.5 mM) and amino acid or its analog. After 2 – 4 days incubation at 37 °C the differences in colony size or colony number between the TG1 strain with hybrid plasmid and control TG1 strain were registered. The results of experiments are presented in Table 1.

Table 1.

Inhibitors	Concentration in media, μg/ml	Effect on the growth of TG1 strain having plasmid pYGAZH
Proline	30000	No
3,4-Dihydroproline	23	R
Isoleucine	18000	No
DL-Thiaisoleucine	1	R
o-Methylthreonine	6	No
L-Serine	2800	No
DL-Serine	3600	No
DL-Serine hydroxamate	140	No
DL-o-Methylserine	3200	R
4-Azaleucine	45	R
6-Diazo-5-oxo-L-norleucine	15	No
Valine	7	R
Methionine	38000	No
Norleucine	500	R
Cysteine	1600	No
Homoserine	1000	No
DL-β-Hydroxy-norvaline	80	No

L-Aspartic acid β-hydroxamate	100	No
Arginine	4300	No
Lysine	5000	No
S-(2-Aminoethyl)cysteine	0.75	No
Histidine	3000	No
L-Histidine hydroxamate	200	No
DL-1,2,4-Triazole-3-alanine	80	No
Phenylalanine	13000	No
p-Fluorophenylalanine	6	No
L-o-Fluorophenylalanine	1.7	R
DL-o-Fluorophenylalanine	6	R
Tryptophan	12500	No
DL-4-Fluorotryptophan	0.1	No
4-Methyltryptophan	0.25	No
7-Methyltryptophan	100	No
DL-a-Methyltrypptophan	400	No
m-Fluoro-DL-tyrosine	0.5	No

No - no differences compare to the control strain
R - more colonies or colony size

Example 3: Production of threonine by a strain having plasmid pYGAZH.

The threonine producing strain VL2054 was transformed by the plasmid pYGAZH carried the b2682 and b2683 genes under the control of P_{lac} UV5 promoter. Obtained strain was named VL2054(pYGAZH). The strain VL2054 is derivative of the strain VKPM B-3996 and carried on its chromosome:

a) the integrated threonine operon under the control of P_R promoter

b) wild type *rhtA* gene

c) the inactivated chromosomal gene encoding transhydrogenase (*tdh* gene) and inactivated kanamycin resistant gene (*kan*) gene in the Tn5 (tdh::Tn5, Kan^S)

d) mutation $ilvA_{442}$.

The strain VL2054 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia 113545, Moscow, 1 Dorozhny proezd, 1) on January 30, 2001 under accession number VKPM B-8067.

The 5 colonies of each strain VL2054, strain VL2054(pΔlacZ) as a control strain contained plasmid without insertion and VL2054(pYGAZH) were suspended in 2 ml of minimal medium ($(NH_4)_2SO_4$ – 11 g/l; NaCl – 0.4 g/l; $MgSO_4$ – 0.4 g/l; K_2HPO_4 - 1 g/l; $FeSO_4$ - 10 mg/l; $MnSO_4$ - 10 mg/l; thiamin – 0.1 mg/l; yeast extract – 0.5 g/l; glucose - 40 g/l; ampicilline - 300 mg/l if necessary) in 20-ml test tubes and were incubated overnight with aeration at 32 °C. The 0.2 ml of each night culture was

transferred to the three 20-ml test tubes with 2 ml of fresh medium for fermentation with or without IPTG and cultivated at 32 °C for 48 or 72 hours with rotary shaker.

Fermentation medium composition:

$(NH_4)_2SO_4$	22 g/l
NaCl	0.8 g/l
$MgSO_4$	0.8 g/l
K_2HPO_4	2 g/l
$FeSO_4$	20 mg/l
$MnSO_4$	20 mg/l
Thiamin	0.2 mg/l
Yeast extract	1 g/l
$CaCO_3$	30 g/l
Glucose	80 g/l
Ampicilline	300 mg/l, if necessary
IPTG	0.5 mM, if necessary

After cultivation the plasmid stability and optical absorbance of the medium at 540 nm were determined by conventional methods. Accumulated amount of threonine in the medium was determined by thin layer chromatography (TLC). Liquid phase composition for TLC was as follows: isopropanol - 50 ml, acetone - 50 ml, NH_4OH (30 %) - 12 ml, H_2O - 8 ml. The results are shown in Table 2. As it is seen, the hybrid plasmid pYGAZH improved the threonine accumulation by the threonine producing strain VL2054.

Table 2.

VL2054 with plasmid	IPTG	48 hours			72 hours		
		OD_{540}	Thr, g/l	Thr/OD	OD_{540}	Thr, g/l	Thr/OD
no	-	19	5.2	0.27	26	9.1	0.35
	+	21	4.1	0.20	29	7.8	0.27
pΔlacZ	-	20	6.4	0.32	24	9.1	0.40
	+	15	3.5	0.23	24	7.2	0.30
pYGAZH	-	17	5.7	0.34	24	9.7	0.40
	+	21	9.8	0.47	23	15.5	0.67

Example 4: Production of valine by a strain having plasmid pYGAZH.

The valine producing strain H-81 was transformed by the plasmid pYGAZH carried the b2682 and b2683 genes under the control of P_{lac} UV5 promoter. The strain H-81 has been deposited in the Russian National Collection of Industrial

Microorganisms (VKPM) (Russia 113545, Moscow, 1 Dorozhny proezd, 1) on January 30, 2001 under accession number VKPM B-8066.

The 5 colonies of each strain H-81, H-81(pΔlacZ) as a control strain contained plasmid without insertion and H-81(pYGAZH) were suspended in 2 ml of minimal medium ($(NH_4)_2SO_4$ - 18 g/l, K_2HPO_4 - 1.8 g/l, $MgSO_4$ - 1.2 g/l, thiamin - 0.1 mg/l, yeast extract – 0.5 g/l, glucose - 60 g/l, ampicilline - 300 mg/l, if necessary) in 20-ml test tubes and were incubated overnight with aeration at 32 °C. The 0.2 ml of each night culture was transferred to the three 20-ml test tubes with 2 ml of fresh medium for fermentation with or without IPTG and cultivated at 32 °C for 48 or 72 hours with rotary shaker.

Fermentation medium composition:

$(NH_4)_2SO_4$	18 g/l,
K_2HPO_4	1.8 g/l,
$MgSO_4$	1.2 g/l,
$CaCO_3$	20 g/l,
Thiamin	0.1 mg/l,
Glucose	60 g/l,
Ampicilline	300 mg/l, if necessary
IPTG	0.5 mM, if necessary

After cultivation the plasmid stability and optical absorbance of the medium at 540 nm were determined by conventional methods. Accumulated amount of valine in the medium was determined by TLC. Liquid phase composition for TLC was as follows: isopropanol - 80 ml, ethylacetate - 80 ml, NH_4OH (30 %) - 15 ml, H_2O - 45 ml. The results are shown in Table 3. As it is seen, the hybrid plasmid pYGAZH improved the valine accumulation by the valine producing strain H-81.

Table 3.

H-81 with plasmid	IPTG	48 hours			72 hours		
		OD_{540}	Val, g/l	Val/OD	OD_{540}	Val, g/l	Val/OD
No	-	34	11,6	0,34	32	10,3	0,32
	+	34	11,7	0,34	30	10,1	0,34
pΔlacZ	-	34	10,5	0,31	30	10,0	0,33
	+	20	7,8	0,39	25	9,0	0,36
pYGAZH	-	29	10,5	0,36	31	12,8	0,41
	+	22	10,8	0,49	23	12,3	0,53

Reference Example 1: Production of L-proline by an *ilvA* deficient L-proline producer.

The cells of wild type strain *E. coli* K12 (VKPM B-7) was treated with a mutagen, N-methyl-N'-nitro-N-nitrosoguanidine (0.1 mg/ml), for 20 min at 37°C, washed and plated on minimal agar medium M9 supplemented with 1.25 mg/ml tryptone, 10 mg/ml L-proline and 0.05 mg/ml 2,3,5-triphenyltetrazolium chloride. Most colonies arisen after 3 day of incubation at 37°C were colored red. A few colonies, which could not oxidize L-proline, were white. One of such colonies was used as a parent for obtaining mutants resistant to proline analogs (3,4-dehydroxyproline and azetidine-2-carboxylate) which were added into M9 agar medium in concentration of 2 mg/ml each.

Some of mutants arisen could produce L-proline. The best L-proline producer 702 was treated with a P1 bacteriophage grown on cells of the strain TG1 in which the gene *ilvA* was disrupted by the insertion of chloramphenicol (Cm) resistance (Cm^r) gene. One of obtained Cm resisitant transductant, 702ilvA, which turned to be L-isoleucine auxotroph, was much more effective L-proline prouducer than the L-isoleucine prototrophic parent strain 702 (Table 4). The fermentation medium contained 60 g/l glucose, 25 g/l ammonium sulfate, 2 g/l KH_2PO_4, 1 g/l $MgSO_4$, 0.1 mg/l thiamine, 50 mg/l L-isoleucine and 25 g/l chalk (pH 7.2). Glucose and chalk were sterilized separately. 2 ml of the medium was placed into test tubes, and inoculated with one loop of the tested microorganisms, and the cultivation was carried out at 37°C for 2 days with shaking.

Table 4

Strain	Phenotype	Accumulation of L-proline (g/l)
K12 (VKPM B-7)	Wild type	<0.1
702 (VKPM B-8011)	Defective L-proline degradation, resistance to proline analogs	0.5
702ilvA (VKPM B-8012)	Defective L-proline degradation, resistance to proline analogs, L-isoleucine auxotroph, Cm^r	8.0

The strains 702 and 702ilvA have been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) under the accession number VKPM B-8011 and VKPM B-8012, respectively, since July 25, 2000.

Example 5: Production of proline by a strain having plasmid pYGAZH.

The proline producing strain *E.coli* 702ilvA was transformed by the plasmid pYGAZH carried the b2682 and b2683 genes under the control of P_{lac} UV5 promoter.

The 5 colonies of each strain 702ilvA, 702ilvA(pΔlacZ) as a control strain contained plasmid without insertion and 702ilvA(pYGAZH) were suspended in 2 ml of minimal medium ($(NH_4)_2SO_4$ - 18 g/l, K_2HPO_4 - 1.8 g/l, $MgSO_4$ - 1.2 g/l, thiamin - 0.1 mg/l, yeast extract – 0.5 g/l, glucose - 60 g/l, isoleucine – 50 mg/l, ampicilline - 300 mg/l, if necessary) in 20-ml test tubes and were incubated overnight with aeration at 32 °C. The 0.2 ml of each night culture was transferred to the three 20-ml test tubes with 2 ml of fresh medium for fermentation with or without IPTG and cultivated at 32 °C for 40 hours with rotary shaker.

Fermentation medium composition:

$(NH_4)_2SO_4$	18 g/l,
K_2HPO_4	1.8 g/l,
$MgSO_4$	1.2 g/l,
$CaCO_3$	20 g/l,
Thiamin	0.1 mg/l,
Glucose	60 g/l,
Isoleucine	50 mg/l
Ampicilline	300 mg/l, if necessary
IPTG	0.5 mM, if necessary

After cultivation the plasmid stability and optical absorbance of the medium at 540 nm were determined by conventional methods. Accumulated amount of proline in the medium was determined by TLC. Liquid phase composition for TLC was as follows: ethanol - 80 ml, NH_4OH (30 %) - 5 ml, H_2O - 25 ml. The results are shown in Table 5. As it is seen, the hybrid plasmid pYGAZH improved the proline accumulation by the proline producing strain 702ilvA.

Table 5.

702ilvA with plasmid	IPTG	40 hours		
		OD_{540}	Pro, g/l	Pro/OD
No	-	25	4,0	0,16
	+	23	4,1	0,18
pΔlacZ	-	24	5,3	0,22
	+	22	5,0	0,23
PYGAZH	-	21	5,0	0,24
	+	23	10,6	0,46

Reference Example 2: Production of L-leucine by an *ilvE* deficient L-leucine producer.

The cells of wild type strain *E. coli* K12 (VKPM B-7) was treated with a mutagen, N-methyl-N'-nitro-N-nitrosoguanidine (0.05 mg/ml), for 20 min at 37°C, washed 4 times with physiological solution and plated on minimal agar medium M9 supplemented with 4.0 mg/ml DL-4-azaleucine. The plates were incubated for 5 days at 37°C. Colonies appeared on the plates were picked up and purified by streaking on the L-agar plates. One of the obtained mutant resistant to DL-4-azaleucine was used for induction of double L-isoleucine and L-valine auxotrophy. The numerous amount of double auxotrophs, requiring L-isoleucine and L-valine for growth, were obtained. It was shown that double L-isoleucine and L-valine auxotrophy was caused by mutation in the *ilvE* gene. Among the obtained double auxotrophs, the best L-leucine producer, strain 505 producing 1.8 g/l of L-leucine, has been selected. The fermentation medium contained 60 g/l glucose, 25 g/l ammonium sulfate, 2 g/l KH_2PO_4, 1 g/l $MgSO_4$, 0.1 mg/l thiamine, 100 mg/l L-isoleucine, 100 mg/l L-valine and 25 g/l chalk (pH 7.2). Glucose and chalk were sterilized separately. 2 ml of the medium was placed into test tubes, and inoculated with one loop of the tested microorganisms, and the cultivation was carried out at 37°C for 2 days with shaking.

The strain *E.coli* 505 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia 113545, Moscow, 1 Dorozhny proezd, 1) on May 14, 2001 under accession number VKPM B- 8124.

Example 6: Production of leucine by a strain having plasmid pYGAZH.

The leucine producing strain *E.coli* 505 was transformed by the plasmid pYGAZH carried the b2682 and b2683 genes under the control of P_{lac} UV5 promoter.

The 20 colonies of each strain 505, 505(pΔlacZ) as a control strain contained plasmid without insertion and 505(pYGAZH) were transferred by one loop of culture in 20-ml test tubes with L-broth with or without ampicilline and were incubated overnight with aeration at 32 °C. The 0.1 ml of each night culture was transferred into the 20-ml test tubes (inner diameter 22 mm), suspended in 2 ml of medium for fermentation with or without IPTG and cultivated at 32 °C for 72 hours with rotary shaker.

Fermentation medium composition:

$(NH_4)_2SO_4$	15 g/l,
K_2HPO_4	1.5 g/l,
$MgSO_4 \times 7H_2O$	1.0 g/l,
$CaCO_3$	20 g/l (sterilized separately),
Thiamin	0.1 mg/l,
Glucose	60 g/l (sterilized separately),
Isoleucine	0.3 g/l
Valine	0.3 g/l
Ampicilline	150 mg/l, if necessary
IPTG	0.5 mM, if necessary

After cultivation the plasmid stability was determined by conventional method. Accumulated amount of leucine in the medium was determined by TLC. Liquid phase composition for TLC was as follows: isopropanol - 80 ml, ethylacetate – 80 ml, NH_4OH (30 %) - 25 ml, H_2O - 50 ml. The results are shown in Table 6. As it is seen, the hybrid plasmid pYGAZH improved the leucine accumulation by the leucine producing strain 505.

Table 6.

505 with plasmid	IPTG	72 hours Leu, g/l
No	-	1,8
	+	2,0
pΔlacZ	-	1,8
	+	2,0
pYGAZH	-	2,0
	+	2,8

Reference Example 3: Production of L-methionine by L-methionine producer resistant to norleucine.

The plasmidless threonine and leucine deficient strain *E. coli* C600 was used as a parental strain. At first, the Leu^+ variants of *E. coli* C600 strain was obtained by transduction of phage P1 grown on *E. coli* K-12 strain. Then, after treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) the mutant strain 44 resistant to 8 g/l of L-homoserine has been obtained. The strain 44 is L-threonine-deficient, resistant to high concentrations of L-homoserine. The strain 44 has been deposited in Russian National Collection of Industrial Microorganisms (VKPM) under the accession number VKPM B-2175.

Then, the strains, which are the mutants resistant to a methionine analog, norleucine, was induced from strain 44 by mutagenesis using NTG. The cells of night culture grown in L-broth were spun down and resuspended in physiological solution (0.9% NaCl) containing 50 μg/ml of NTG. After 30 min of exposure with NTG at 37°C the cells were spun down, washed 4 times with physiological solution and plated on the minimal agar medium M9, containing 0.5 mg/ml of threonine and 2.5 mg/ml or 5.0 mg/ml of norleucine. The plates were incubated for 5 days at 37°C. Colonies appeared on the plates were picked up and purified by streaking on the L-agar plates. The best L-methionine producer among them was strain 218. Test-tube cultivation of the novel strain 218 carried out at 32°C for 3 days with shaking leads to accumulation in the culture medium 1.1 g/l of L-methionine. As a fermentation medium was used minimal medium containing ammonia sulfate (15.0 g/l), KH_2PO_4 (1.5 g/l), $MgSO_4$ (1.0 g/l), thiamin (0.1 mg/l), glucose (40 g/l), threonine (0.5 g/l), calcium carbonate (20 g/l). Glucose and chalk were sterilized separately.

The strain 218 has been deposited in Russian National Collection of Industrial Microorganisms (VKPM) under the accession number VKPM B-8125 since May 14, 2001.

Further, the phage P1 mediated deletion of ppc gene has been introduced into strain 218 followed by integration of *pycA* gene from *Bacillus subtilis* (Russian patent application 99121636). Resulted strain 218pycA lost resistance to norleucine. Therefore, resistance to norleucine has been imparted to the strain again as described above. The best L-methionine producer among obtained strains was strain *E.coli* 73 which produced about 1 g/l of L-methionine under condition described above.

The strain *E.coli* 73 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia 113545 Moscow 1 Dorozhny proezd, 1) on May 14, 2001 under accession number VKPM B-8126.

Example 7: Production of methionine by a strain having plasmid pYGAZH.

The methionine producing strain *E.coli* 73 was transformed by the plasmid pYGAZH carried the b2682 and b2683 genes under the control of P_{lac} UV5 promoter.

The 5 colonies of each strain 73, 73(pΔlacZ) as a control strain contained plasmid without insertion and 73(pYGAZH) were suspended in 2 ml of minimal medium ($(NH_4)_2SO_4$ - 18 g/l, K_2HPO_4 - 1.8 g/l, $MgSO_4$ - 1.2 g/l, thiamin - 0.1 mg/l,

yeast extract – 10 g/l, glucose - 60 g/l, threonine – 400 mg/l, ampicilline - 300 mg/l, if necessary) in 20-ml test tubes and were incubated overnight with aeration at 32 °C. The 0.2 ml of each night culture was transferred to the three 20-ml test tubes with 2 ml of fresh medium for fermentation with or without IPTG and cultivated at 32 °C for 48 hours with rotary shaker.

Fermentation medium composition:

$(NH_4)_2SO_4$	18 g/l,
K_2HPO_4	1.8 g/l,
$MgSO_4$	1.2 g/l,
$CaCO_3$	20 g/l,
Thiamin	0.1 mg/l,
Glucose	60 g/l,
Threonine	400 mg/l,
Yeast extract	1.0 g/l,
Ampicilline	300 mg/l, if necessary
IPTG	0.5 mM, if necessary

After cultivation the plasmid stability and optical absorbance of the medium at 540 nm were determined by conventional methods. Accumulated amount of methionine in the medium was determined by TLC. Liquid phase composition for TLC was as follows: isopropanol - 80 ml, ethylacetate - 80 ml, NH_4OH (30 %) - 15 ml, H_2O - 45 ml. The results are shown in Table 7. As it is seen, the hybrid plasmid pYGAZH improved the methionine accumulation by the methionine producing strain 73.

Table 7.

73 with plasmid	IPTG	48 hours		
		OD_{540}	Met, g/l	Met/OD
No	-	45	0,7	0,016
	+	42	1,1	0,026
pΔlacZ	-	45	1,0	0,022
pYGAZH	-	48	0,9	0.019
	+	46	1,3	0,028

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What is claimed is:

1. An L-amino acid producing bacterium belonging to the genus *Escherichia* wherein L-amino acid production by said bacterium is enhanced by enhancing activities of proteins as defined in the following (A) or (B) , and (C) or (D) in a cell of said bacterium:

(A) a protein which comprises the amino acid sequence shown in SEQ ID NO:3 in Sequence listing;

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO:3 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to L-amino acids and/or its analogs;

(C) a protein which comprises the amino acid sequence shown in SEQ ID NO:5 in Sequence listing;

(D) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO:5 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to L-amino acids and/or its analogs.

2. The bacterium according to the claim 1, wherein said activities of proteins as defined as (A) or (B) and (C) or (D) are enhanced by transformation of said bacterium with DNA coding for protein as defined in (A) or (B), and (C) or (D), or by alteration of expression regulation sequence of said DNA on the chromosome of the bacterium.

3. The bacterium according to the claim 2, wherein the transformation is performed with a multicopy vector.

4. A method for producing L-amino acid, which comprises cultivating the bacterium according to any of claims 1 to 3 in a culture medium and collecting from the culture medium L-amino acid to be produced and accumulated.

5. The method according to claim 4, wherein L-amino acid is L-threonine.

6. The method according to claims 5, wherein the bacterium has enhanced expression of threonine operon.

7. The method according to claim 4, wherein L-amino acid is L-valine.

8. The method according to claims 7, wherein the bacterium has enhanced expression of *ilv* operon.

9. The method according to claim 4, wherein L-amino acid is L-proline.

10. The method according to claims 9, wherein the bacterium has enhanced expression of genes for proline biosynthesis.

11. The method according to claim 4, wherein L-amino acid is L-leucine.

12. The method according to claims 11, wherein the bacterium has enhanced expression of *leu* operon.

13. The method according to claim 4, wherein L-amino acid is L-methionine.

14. The method according to claims 13, wherein the bacterium has enhanced expression of genes *met* regulon.

Abstract of disclosure

There is provided a method for producing L-threonine, L-valine, L-proline, L-leucine and L-methionine using bacterium belonging to the genus *Escherichia* wherein L-amino acid productivity of said bacterium is enhanced by enhancing an activity of proteins coded by b2682 and b2683 genes.

Figure 1. Scheme for construction plasmid pΔlacZ.